COMPARATIVE SEROLOGY OF SOME BRACHYURAN CRUSTACEA AND STUDIES IN HEMOCYANIN CORRESPONDENCE[1]

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Introduction

Researches in systematics may be conducted by comparing the serum or corresponding body proteins of organisms using serological methods. The underlying principle of serological systematics is that the proteins compared are representative of the organisms producing them in the same sense that their corresponding structures are and for the same general reasons. Of the serological reactions used for such researches the precipitin reaction has undoubted advantages. An extension of the previous precipitin studies in the serological systematics of Crustacea and related problems is the content of this report.

General Materials and Methods

Antigens

The sera of the Crustacea compared in this study were in part provided by Dr. Alan A. Boyden[1] who gathered them during summers over a period of years at various biological stations along the Eastern coast of the United States and elsewhere, namely, Mount Desert Island Biological Laboratory, Salisbury Cove, Maine, 1936; U. S. Bureau of Fisheries Laboratory, Beaufort, North Carolina, 1936; Tortugas Laboratory, Carnegie Institution, Washington; Key West, Florida, 1932, 1934, 1936, 1939; also at the Marine Laboratory, Citadel Hill, Plymouth, England, 1939, 1948.

In addition to the samples of sera, the author had access to Dr. Boyden's original records made at the time of collection, plus the correpondence incidental to the collection and identification of particular samples. These records were of great help in orientation to the work and in providing useful hints and suggestions which saved countless hours when the author did his own field collecting.

Useful serum samples were secured by the writer in the field at Barnegat Bay and Delaware Bay, New Jersey. Samples of European crustacean sera were collected in the Summer of 1948 by the author at the following European biological laboratories: Museum National D'Histoire Naturelle Laboratoire Maritime, Dinard, France; Universite de Paris, Biologie Marine, Laboratoire Arago, Banyuls-sur-Mer, France; and the Stazione Zoologica, Napoli, Italia.

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In general the blood was taken from the crabs by removing the 5th pereiopod and permitting the blood of the organisms to drain into porcelain pans or glass crystallizing dishes. The crabs were held over the collecting pans by means of an ordinary clamp attached to a cross bar supported between two vertical stands. In the case of the long-tailed Crustacea such as crayfish and lobsters, the blood was collected by slitting the abdomen ventrally where it joins the thorax and holding the organism by hand over a large collecting funnel making sure the posterior end of the abdomen was pressed firmly to the lip of the funnel as an insurance against sudden abdominal flexures. It is necessary to occasionally break the jelly-like clots that form at the openings from which the blood is draining in order to obtain good yields from each animal. In those cases where the organisms were small the blood was taken from convenient points within the body cavity by means of needle and syringe. Piercing the body at the base of the appendages usually gave good yields. It is interesting to note here that placing the needle in the heart or in the pericardium gave poorer results than when a sinus some distance from the heart was tapped.

TABLE I

List of the species of Crustacea used as antigens

Acanthocarpus alexandri Stimpson
Callinectes marginatus (Milne-Edwards)
Callinectes sapidus Rathbun
Cancer anthonyi Rathbun
Cancer borealis Stimpson
Cancer irroratus Say
Cancer magister Dana
Cancer pagurus L.
Carcinus maenas L.
Geryon quinquedens Smith
Maia squinado Rondelet (Herbst)
Menippe mercenaria (Say)
Mithrax verrucosus Milne-Edwards
Ocypode albicans Bosc.
Panopeus herbstii Milne-Edwards
Panulirus argus (Latreille)

The collected bloods were permitted to clot and the sera to be expressed. Centrifugation at 2000–3000 r.p.m. for 20 minutes was usually employed to clear the sera. It is feasible to hasten the expression of serum by wrapping the clot in a double thickness of clean, fine-mesh, bolting or cheese cloth and twisting the wrapping by hand. The sera were sterilized by Seitz filtration, bottled in serum vials and stored at $3° \pm 1°$ C.

Table I is a list of the Crustacea whose sera were used as antigens in this work. The identifications of North American Crustacea were made with the works of Pratt (1936), Faxon (1898), and the remarkable publications of Rathbun (1917, 1925, 1930, 1937). The French fauna was checked against Bouvier (1940). The fauna of other European countries was identified in the works of Borradaile (1907), Pesta (1918), and Thiele (1935).

Antisera

All antisera were produced in rabbits. Intravenous and/or subcutaneous routes of injection were employed in the production of antisera. Inasmuch as it is impossible to ascertain at present the degree to which a rabbit will respond to a given amount of antigen, and since this response is perhaps the greatest variable in present day serological studies, what was believed to be minimal amounts to produce a good response were used. Generally 0.25 cc. was given on the first injection followed on alternate days with three 0.5 cc. injections. In every instance good, usable antisera were obtained when the hemocyanins were injected in these doses. In some cases a presensitization technique was employed to improve the strength (i.e. precipitating capacity) of the antisera. These rabbits were given 0.25 cc. of antigen intravenously and then permitted to rest for 30 days. This was followed by a series of 4 subcutaneous injections given on alternate days.

TABLE II

List of antisera

Antiserum	Homologous antigen	Remarks
I–63	*Menippe mercenaria* 36-A	Prepared by Dr. Alan A. Boyden Rutgers University
I–76	*Panulirus argus* 39–1	
I–77	*Panulirus argus* 39–1	
I–78	*Cancer borealis* 3a1	
I–86	*Callinectes sapidus* 47–1	
I–87	*Callinectes sapidus* 36–1	
I–94	*Cancer borealis* 1a	
I–95	*Maia squinado* No. 5	
I–97	*Cancer pagurus* No. 3	
I–98	*Menippe mercenaria* 36–A	
I–99	*Ocypode albicans*	
I–100	*Geryon quinquedens* 36–1	
I–101	*Cancer irroratus* 36–1	
I–105	*Geryon quinquedens* 36–1	
I–106	*Cancer irroratus* 36–1	
I–107	*Acanthocarpus alexandri*	
I–110	*Geryon quinquedens* 39–1	
I–114	*Callinectes sapidus* 47–1	

Table II is a list of antisera prepared and utilized by the author except where specially annotated.

Bleeding the rabbits to secure the antisera was accomplished in either one of two ways. Small samples of blood were withdrawn from the central artery of the ear, by using a number 22 gauge needle and syringe. For complete bleedings blood was withdrawn directly from the heart by cardiac puncture. Size number 18 gauge needles and 50 cc. syringes were used in this latter procedure. All antisera were centrifuged, sterile filtered through Seitz filters, and stored in the refrigerator until used.

Method of testing

The Libby photronreflectometer (1938) was utilized exclusively in the measurement of turbidities developed as a result of the interaction of antigens and antibodies. The technique employed was essentially the same as that described by Boyden and DeFalco (1943). Minor variations in technique as developed by the writer were matters of convenience and did not represent any major changes in their method of making the dilution series (see Figs. 1 and 2) of the antigens, nor in the use of the machine. Two recent papers (Boyden et al., 1947; Bolton et al., 1948) have analyses of the performance of the photronreflectometer and report the conclusion that for white precipitate systems, which include all the precipitin reactions, the instrument is unsurpassed at present in its sensitivity and range of usefulness in studying the characteristics of precipitates.

For all tests the procedure in which the amount of antiserum is held constant and the amount of antigen is varied was employed. The reacting cells of the photronreflectometer have a 2 cc. operating level and this volume was used in all testing. Final volume for each antigen dilution was always 1.7 cc. to which 0.3 cc. of immune serum was added to make up the 2 cc. volume. Turbidities (i.e. galvanometer readings) inherent in the fluid of the antigen dilution, and those due to dirt or blemishes on the glass of the reacting cells plus the turbidity characteristic for 0.3 cc. of each antiserum used were deducted from the total turbidity developed in each reacting cell. For these reasons the resultant turbidities can be considered as those due to the interaction of antigen and antibody.

The range of antigen dilutions regularly employed was between 4000 and 1 gamma of protein per cubic centimeter or solution, or in terms of dilutions of protein from 1 : 250 in doubling series to 1 : 1,024,000. When necessary the range of antigen dilution was extended. For the sake of convenience in plotting the results of the reaction on graphs, the antigen dilution cells were assigned numbers in a chronological sequence with cell No. 1 containing the greatest concentration of antigen. Figure 1 shows graphically a typical dilution sequence.

For all of the work in this paper the precipitin turbidities were those developed during 20 minutes incubation in a dry-air incubator maintained at 38° C.

The titration curve

The graph of the turbidities developed for serological tests is usually made in this laboratory with the turbidities plotted as the ordinate and the antigen dilution as the abscissa with the greatest concentration of antigen nearest the x-y axes intersect. The turbidities rise to a maximum, then decline again to a minimum following generally a normal distribution curve. By assigning unit distances between the geometrically changing antigen concentration values along the abscissa, the curves as plotted are more or less symmetrical. Skewness toward the region of antigen excess is quite common. Variations in the amount of kurtosis have also been observed. The amounts and kinds of both antigens and antibodies in the solutions combine to provide many variations of this type of frequency distribution. Figure 2 represents an idealized titration curve. The numerical value used to characterize any one particular curve for comparative purposes is that obtained by summating the turbidities over the whole reaction range. This value is proportional

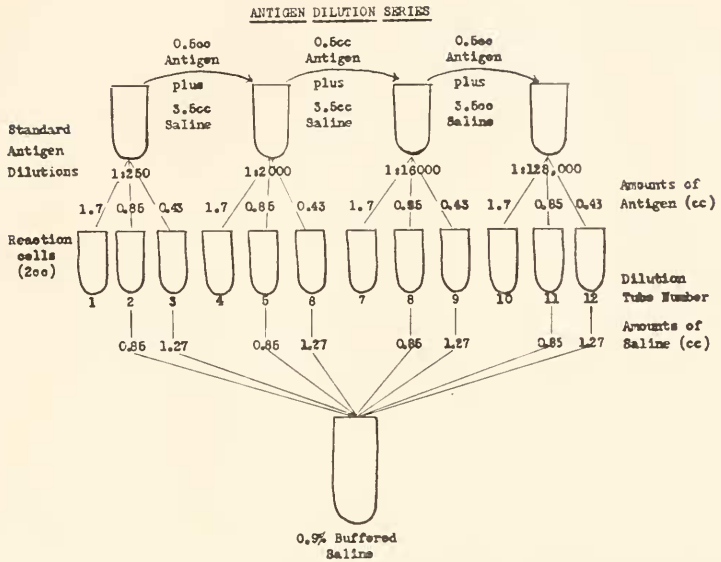

FIGURE. 1. A diagram of the technique used in preparing a typical doubling-dilution series of a given antigen for use in the Libby Photronreflectometer. The initial standard antigen dilution (1:250) is prepared directly from a serum of known protein concentration. The subsequent standard antigen dilution tubes are prepared from the first. Each reaction cell has a constant antigen dilution volume of 1.7 cc. to which is added and mixed a constant volume of 0.30 cc. of antiserum (a procedure).

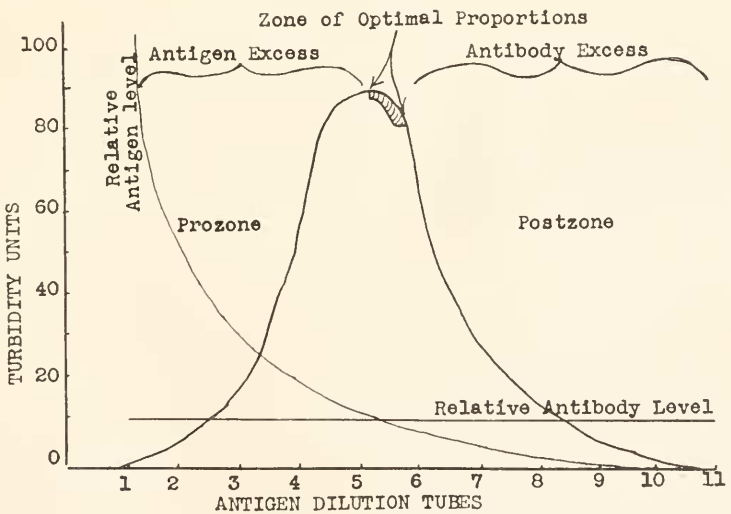

FIGURE 2. A diagrammatic representation of a typical titration curve obtained by using the Libby Photronreflectometer as the turbidimeter, and the technique of reacting varying antigen dilutions with a constant amount of antibody. Included also are appropriate curves to portray the relative antigen and antibody levels in each antigen dilution tube. For these latter curves the turbidity units of the ordinate axis do not apply.

to the area under the curve and provides an easily obtained statistical index of a very complicated biochemical system.

For all titrations the dilution medium was 0.9 per cent NaCl buffered with M/15 phosphate salts (Sorensen's solution) (Evans, 1922) such that the buffer was in a final concentration of M/150. The pH range for the tests was between 7.05 and 7.15.

Antigen Correspondence

The continuous analysis of the properties of both of the primary reagents used in serological work is necessary if the investigations are to be considered critical. This problem is especially pertinent to workers in the field of serological systematics since they must be certain that the sera or proteins or organisms used in their tests are unchanged from the native state, or if changes have occurred because of prolonged storage or other physical or chemical factors, they must be prepared to correct for it.

The sera of Crustacea contain one principal protein, hemocyanin (Allison and Cole, 1940). They are excellent antigens when injected into the rabbit. They are usually considered to be relatively pure systems. However, electrophoretic patterns (Cohn and Edsall, 1943), sedimentation constants (Dawson and Mallette, 1945; Redfield, 1934), and $(NH4)_2SO_4$ precipitation (Bolton, personal communication) all indicate that this single serum protein may be composed of several molecular "species" of hemocyanin. The hemocyanin molecules are large with molecular weight ranging from 300,000 upward to several millions as calculated by Svedberg and his collaborators from data obtained by using the ultracentrifuge methods of sedimentation velocity and sedimentation equilibrium. A glance at almost any titration curve of the hemocyanin antigen-antibody system (Fig. 3) will show irregularities and disturbances in the modality of the plot. Occasionally a titration will show two distinct modes. These variations from a single mode frequency curve have long been considered as evidence of the probable presence of more than a single kind of antigen or antibody in the system. It is quite likely that the above mentioned molecular "species" are responsible for the stimulation of more than a single principal kind of antibody in the rabbit serum. This, of course, does not preclude the possibility that a single molecular "species" could stimulate the production of two or more distinct kinds of antibodies. Until such time as this can be proven, it is simpler to assume that a single kind of antibody is produced against each kind of antigen, and that the appearance of additional modes in the titration curve of any antiserum is due to the reaction of individual molecular "species" in the antigen complex with their homologous antibodies. This assumption is not inconsistent with the observed behavior of antigen-antibody systems.

The serological identification of all proteins resides in their structural peculiarities and in their chemical nature, i.e., the kinds, proportions and arrangement of the amino acids and prosthetic groups all are believed to affect the serological activity of proteins. It is well known that mild treatment both chemical and physical, will alter the nature of proteins (Landsteiner, 1936; Cohn and Edsall, 1943). This fact presents a challenge to all investigators who are using animal proteins as representing the nature of the organisms with which he is working. Studies in serological systematics may require that animal sera be collected and saved, sometimes for a period of years, before numbers of different species sufficient for comparative in-

FIGURE 3. A typical series of precipitin titration curves showing an order of serological relationship among four crabs. The homologous reaction between *Cancer borealis* and the antiserum produced against it exceeds all others. Then the alignment occurs, in the order of their decreasing curve areas, *Callinectes sapidus, Acanthocarpus alexandri,* and *Maia squinado,* which is in accord with accepted systematic relationships.

vestigations are secured. Further, sera frequently have to be collected under field conditions, necessitating the use of preservatives. There are other aspects of the general problem of antigen comparability than those resulting from the chemical action of preservatives which must also be considered before comparisons of animal sera can be made with confidence. Following are the results obtained from attempts to test the affect of some of the variables encountered in the preparation of animal sera for comparative studies in serological systematics.

Physical treatment

Usual laboratory procedures have been to allow the blood to clot and the sera to be expressed for about 24 hours. The sera are then centrifuged at 2000–3000 r.p.m. for 20 minutes, filtered sterile in Seitz filters, bottled, and stored in the refrigerator at 3° ± 1° C. Any or all of these steps could alter the nature of the serum proteins and thus modify their serological activity. Table III summarizes a series of tests performed to examine the effects of treatment in the laboratory. The sample of blue crab serum designated in the table as *Callinectes sapidus* 47–2 represents the sera of 24 large male crabs all bled within one hour. The pooled collection of sera was divided into several parts and given various kinds of physical treatment in the laboratory. All of the antigens were tested against antiserum I–86 (Anti-*C. sapidus* 47–1) which was prepared against a fresh, sterile filtered

sample of crab serum. The antiserum I–86 was powerful and was diluted with 10 parts buffered saline at pH 7.0. Since dilution is known to increase the specificity of an antiserum (Boyden and DeFalco, 1943) under certain conditions it was advantageous to dilute the antiserum in this manner to magnify any differences among the antigens. The dilution factor was so chosen that total turbidities would summate in the vicinity of 300 galvanometer units. A curve area this large tends to minimize variations in results due to errors in experimental techniques. Experimental error is limited to 5 per cent. In addition to the treatments listed in the table attempts were made to lyophilize (freeze-dry) (Florsdorf and Mudd, 1935) parts of this sample, but "boiling" [3] occurred during the course of drying, which denatured the proteins to such an extent that insufficient amounts of them could be restored to conduct comparative studies.

TABLE III

Effect of laboratory handling on antigen reactivity

Antiserum I–86 Anti *C. sapidus* 47–1 (1 + 10) × *C. sapidus* 47–2

Treatment	Area	% Change
Centrifuged, filtered	279	0
Centrifuged, unfiltered	283	1
Uncentrifuged, unfiltered	287	3
Frozen, centrifuged	287	3
Frozen, uncentrifuged	290	4
Room temperature, centrifuged, filtered	275	1
Room temperature, centrifuged, merthiolate	285	2

The homologous antigen *Callinectes sapidus* 47–2 was centrifuged and sterile filtered before it was used as an antigen. Changes due to above listed treatments appear to be negligible.

It is realized that any tests involving comparisons with only a single antiserum prepared from but one kind of the possible antigen types present but a minimum of data on the comparability of antigens. Moreover, in the type chosen (Table III, explanation), it still is possible that the unfiltered, and the frozen samples could have constituents not possessed by the filtered samples. That there was a high degree of correspondence among all of the antigens tested is testimony to the fact that the unfiltered, and frozen samples contain corresponding antigens to those in the filtered material, also that filtration does not significantly reduce the quantity of such antigens nor the quality of their reacting (combining) capacity. For the short period of time (24 hours) involved between the beginning and end of the processing, the sample held at room temperature showed no difference from the others. A retesting of the room temperature sample seven days later also revealed no significant change in activity.

[3] This is the bubbling which occurs when the rate of sublimation of the frozen material undergoing desiccation is slow and thawing occurs at the inside glass surface of the containers because of the transfer of atmospheric heat to the frozen solid while the whole system is under vacuum. The relationships between the rate of heat intake from the atmosphere at the exterior glass surface of the containers, the rate of heat loss at the evaporating surfaces of the product and the rate of escape of water vapor from the product to the condenser is apparently upset.

Effect of cold storage

The question of antigen comparability is bound to the problems related to the storage of sera for prolonged periods. In order to examine adequately the effects of storage it was necessary to establish whether or not significant alterations occurred in samples of sera of various ages. Table IV summarizes the results of these tests. It is readily seen that no demonstrable immunological alteration occurred between the samples one day old (22 hours) and the other older samples.

TABLE IV

Antigen comparability

Sample	Number of individuals	Per cent protein	Whole curve area	Per cent deviation	Age of sample when tested
47–3	12	7.04	280.1	−0.3	22 hours
47–2	12	7.00	278.8	−0.1	1 month
*47–1	24	7.39	279.1	0.0	4 months
36–1	5	5.05	281.8	−0.9	11 years

Table showing the lack of change in serum samples of the blue crab, *Callinectes sapidus*, when tested with an antiserum against one of them (*). Samples stored at 3° ± 1° C.

The fact that the eleven year old sample number 36–1 of *C. sapidus* possessed a precipitinogen activity equal to the very fresh sera of this species is very surprising and important. This old serum has been kept at refrigerator temperatures practically continuously since it was collected in 1936. The amount of alteration that goes on in these serum proteins, if any occurs, must be very slight not to be detected by immunological testing. All the samples listed in Table IV represent pooled samples of the sera of five or more animals. It is conceivable, however, that individual variability of the sera does exist and that the pooling nullifies this variation, presenting for test a more or less "common denominator" kind of serum to the testing antiserum.

Serological variation

Interesting evidence of the stability and lack of variability among various samples of sera of the same species of spiny lobsters, *Panulirus argus*, is illustrated in Table V. Here samples collected over a period of years (1932–1939) were tested against an antiserum made to one of them. The test antigens were selected to reveal the differences due to aging, differences due to sex, and differences between single specimen samples and pooled samples containing the sera of more than one individual. The amount of difference in the serological reactivity among these different categories was negligible. In no instance did the variations in the whole curve areas exceed the experimental error of the method of testing. This is a remarkable fact, considering the length of time these sera have been stored; the youngest sample being nine years old at the time of testing, the oldest sample being fifteen years old.

Considering the sensitivity of the precipitin tests to slight alterations in chemical structure, the lack of variation in the amount and kind of reactivity demonstrated in the data of Tables IV and V is testimony first to the care in the laboratory prepa-

TABLE V

Antigen comparability

Sample	Number of individuals	Per cent protein	Whole curve area	Per cent deviation
32–1	"Pooled"	6.07	345.3	−0.2
32–2	"Pooled"	6.52	345.6	−0.1
34–C	1	4.55	351.6	1.5
34–D	1	5.33	340.8	−1.4
34–E	1	2.66	357.0	2.9
34–F	1	7.66	355.7	2.8
34–G	1	3.69	343.7	−0.6
36–1	1	5.31	345.2	−0.2
36–3 & 4	2	10.05	353.6	2.5
36–5 & 6	2	7.44	350.6	1.3
36–10	1	6.07	354.5	2.5
36–11	4	6.52	351.3	1.5
*39–1	5	4.59	345.9	0.0
39–3	9	6.02	349.4	0.2

Table showing the high degree of serological similarity among different samples of the spiny lobster, *Panulirus argus* collected between 1932 and 1939, and tested with an antiserum against one of them (*). Tests were conducted in 1947.

ration of the sera, and the use of a correct method for maintaining useful samples for long periods of time, and second, a testimony to the durability and stability of hemocyanin proteins when collected and preserved in the manner described above.

Color changes

Hemocyanin proteins possess a copper radical and in the oxidized state and in vitro have a blue to green color when in solution. Occasionally, variations have been noted in the color of the sera after they have been sterile filtered and bottled. The most frequent variation is a change from the blue-green color to dark brown. This change is due in part to the free carbon on the Seitz pads which forms there as a result of over-heating during sterilization of the filter, and also to chemical changes in the proteins themselves since the color change has been noted not only during the filtration processing but also in some samples previous to filtering. The brownish color does not appear to play any role in altering the reactivity of the sera since these samples compare favorably with others of the same species possessing the blue-green color.

If a sample of hemocyanin serum is contaminated with bacteria, these organisms flourish and the blue-green, oxidized condition of the serum changes to a colorless liquid, with a cloudy suspension of bacteria. If such sera are kept in the refrigerator, even though contaminated, no recognizable alteration occurs in the activity of the dissolved protein when tested against an antiserum. Removal of the bacteria by filtration or centrifugation or both quickly restores the oxidized condition of the serum. Its serological behavior seems not to be altered even when the serum has been stored in the contaminated state in the refrigerator for prolonged periods.

Precipitates in vials

Another phenomenon that has been seen to occur in sterile, bottled sera is the appearance of precipitates in the vials. In some instances the amount of material coming out of solution has been considerable. The supernatants of many of these vials were examined and found still to contain sufficient protein in solution to warrant testing for comparability with nonprecipitated samples of the same species. Surprisingly enough the still soluble fractions of the total proteins in the antigens apparently possessed all of the reactivity characteristic for the serum. This would indicate that the precipitate is denatured protein which has not undergone any appreciable decomposition. The presence of large amounts of free amino acids and peptides in solution above the precipitates would present free radicals that might combine with the antiserum, blocking the reaction and causing some difference in the amount of reaction observed to occur between the still soluble protein and the antiserum. This is not the case with these systems. The precipitates, moreover, still possess the capacity to combine with antisera, as saline suspensions of them readily reveal. The antigen precipitates go back into solution readily using dilute alkali, but not in saline solutions up to 1.7 per cent NaCl.

Serological Systematics of Some Crustacea

Using the procedures described above, it thus appears feasible to collect and store the sera of Crustacea for long periods of time (15 years in the data given) without significant alteration in their specific properties. Comparative studies of the various species of Crustacea become in effect analysis of the biochemical nature of the organisms concerned based on the quantitative comparison of the nature of their serum proteins, as reflected by turbidities developed in the antigen-antibody reaction.

Erhardt (1929) reviewed the early work done on serological systematics among the Crustacea. Some of these early investigators obtained results which do not agree with systematic classifications based on morphological and embryological data. In one instance Nuttall and Graham-Smith, each using the same antiserum, compared exactly the same organisms and obtained very different results. In most other instances the serological work generally agreed with well established classifications. Most all of these early tests were done using the interfacial or "ring" test method for comparisons. The correspondence of techniques among the various workers ended there because the antigens were not standardized and because results of tests based on antiserum titers and those based on antigen titers cannot be directly compared.

Boyden (1942) pointed out the deficiencies and inadequacies in these early serological investigations and in two papers (1939, 1943) presented the first really quantitative study in the serological systematics of the Crustacea. He compared representatives of five families of the Brachyura and two species of the Macrura. It was in the later paper that the concept of the "serological yardstick" was introduced. The relationships of the sera of species of the same genus, and genera of the same family, and different families of the tribe Brachyura were remarkably consistent in their serological values. All of his tests were made using the photronreflectometer and standard procedures as outlined by Boyden and De-

Falco (1943). The inconsistencies apparent in earlier investigations by other workers were not observed to occur in these studies.

The availability of a larger and more representative group of Crustacea made an extension of Boyden's work possible. Table VI summarizes the serological comparisons made. It should be pointed out that the values given are averaged from two or more tests. The generally higher interspecific, intergeneric and interfamily relationships than those reported by Boyden seem to be due to the use of the presensitization technique to produce more powerful and less discriminating antisera.

From Table VI it is apparent that the antisera differ in their capacities to discriminate among the families of Brachyura. It appears that the Portunidae, Xanthidae and Cancridae are more closely related to each other than they are to the Ocypodidae, Calappidae and the Majidae. Of special interest is the species *Geryon quinquedens* here listed as a member of the family Goneplacidae, in accordance with Rathbun's classification (1937). She concluded that Goneplacidae were closely related to the Xanthidae. Bouvier (1940) places Geryon in the family Xanthidae. The serological tests indicate a degree of correspondence for *Geryon* which is approximately the same in heterologous reactions as for the xanthid species. Further critical testing is necessary to definitely establish the affinities of *Geryon*.

Table VI

The relative relationship among Crustacean species representing seven families in the tribe Brachyura

Antisera	Antigens (Relationship in per cent)												
	Callinectes sapidus (Portunidae)	*Callinectes marginatus* (Portunidae)	*Carcinus maenas* (Portunidae)	*Cancer borealis* (Cancridae)	*Cancer irroratus* (Cancridae)	*Cancer pagurus* (Cancridae)	*Cancer anthonyi* (Cancridae)	*Panopeus herbstii* (Xanthidae)	*Menippe mercenaria* (Xanthidae)	*Ocypode albicans* (Ocypodidae)	*Acanthocarpus alexandri* (Calappidae)	*Geryon quinquedens* (Goneplacidae)	*Maia squinado* (Majidae)
Callinectes sapidus (Portunidae)	100	78	46	33	44	37	33	24	48	18	12	38	8
Cancer borealis (Cancridae)	29	22		100	75	54		20	42	9	8	22	9
Cancer irroratus (Cancridae)	49	46	33	78	100	73	72	30	35	43	10	31	30
Menippe mercenaria (Xanthidae)	27		21	24	21	16	24	37	100	8		27	3
Ocypode albicans (Ocypodidae)	30		40	33		34		39	34	100	39	6	33
Acanthocarpus alexandri (Calappidae)	12		13	15		15				18	100	14	2
Geryon quinquedens (Goneplacidae)	15			13					15		8	100	9
Maia squinado (Majidae)	21			16					13	13	8		100

From Table VI it is readily seen also that species within a given genus react with each other to a greater extent than with any other organisms.

The problem of establishing in detail interfamily relationships among Crustacea by serological methods is one that would entail the production of large numbers of antisera in an attempt to secure serological reagents which are sufficiently powerful to react significantly with the more distant families and at the same time discriminate among the representatives of these families sufficiently to establish a verifiable order of relationship.

Summary

Serological Systematics is a branch of Serology concerned primarily with the classification of organisms. The taxonomic characters usually concerned are the serum proteins of organisms. The natural relationships obtained are those revealed by an antiserum in combination with its homologous antigen and various heterologous antigens which react in proportion to their degrees of correspondence to the homologous antigen. Many factors influence the antigen-antibody reaction. A few conditions which are of importance to studies in systematic serology have been investigated and the results given.

Antigens tested for comparability under a variety of the circumstances met in ordinary laboratory handling such as freezing, filtration and centrifugation, showed no significant deviation from each other in their serological activity. Age, within the limits stated, was shown to have no effect on the serological activity of serum antigens which are sterile filtered and stored just above freezing. Antigens in cold storage for as long as 15 years had the same activity as freshly prepared samples. Pooled serum antigens showed no serological differences from the sera of individuals. No differences were demonstrated in sera due the sex of the organisms. For cold stored antigens color changes in the vials did not indicate alteration of the reactivity of the proteins. The remaining soluble portions of protein in vials showing apparently spontaneous precipitation of the protein gave the same reactivity as freshly prepared antigens. Bacterial contamination, if not permitted to endure too long, and if kept under refrigeration, does not alter the proteins significantly. The reconstitution of lyophilized hemocyanin sera for serological testing was not successful.

The studies in systematic serology have been extended to include new families of decapod Crustacea. For the species of Brachyura tested it appears that the families Portunidae, Xanthidae, and Cancridae are more closely related to each other than all of them are to the Ocypodidae, Calappidae and Majidae.

Further studies into the conditions which may modify the serological reactivity of proteins are needed. Continuing investigations, examining both the validity of the methods used in measuring antigen-antibody reactions and the methods used in the preparation of these primary serological reagents, are necessary components of truly critical studies in serological systematics.

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